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The Effect of Omega-3 Polyunsaturated Fatty Acids Derived from Various Sources on Bone Metabolism in Growing Female Rats

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ABSTRACT

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Bone loss may be lessened if bone mass and strength are increased by optimizing nutrition intervention. Omega-3 polyunsaturated fatty acids (ω-3 PUFAs) have been suggested to improve bone mineralization and microarchitecture by affecting mineral balance and lipid peroxidation. However, the sources of the ω-3 PUFA differ in the types of ω-3 PUFA, ratios, and structural form. Therefore, the study objective was to determine the effect of ω-3 PUFAs from different sources on bone mineral and microarchitecture and explore potential associations. Growing (28 day) female Sprague-Dawley rats were randomly assigned (n=10/group) to a high fat 12% (weight) diet consisting of either corn oil (CO) control or the ω-3 fatty acid rich flaxseed (FO), krill (KO), menhaden (MO), salmon (SO), or tuna (TO). After 8-weeks of feeding, femur and tibia were collected. Bone morphometry, bone mineralization, and microarchitecture were measured. Bone mineralization and microarchitecture were measured by dual-energy x-ray absorptiometry (DEXA) and micro-computed tomography (μCT), respectively. Bone strength was measured using a 3-point bending test. Mineral balance was determined by inductively coupled plasma optical emission spectrophotometry. Bone turnover markers were measured by standard enzyme immunoassay (EIA). Lipid peroxidation was determined by measuring the amount of thiobarbituric acid reactive substances (TBARS) using EIA. In the tibiae, rats fed TO had longer (P<0.001) tibiae than rats fed CO, FO, KO, and MO. Tibiae bone mineral content was greater in rats fed TO or SO (P<0.001) than CO-fed rats. Tibiae bone mineral density in TO-fed rats was higher (P=0.006) compared to CO-fed rats. Trabecular microarchitecture in the femur and tibiae showed rats fed FO or MO had improved bone microarchitecture compared to rats fed CO or SO. There were no significant differences in bone strength. Serum osteocalcin was higher (P=0.03) in rats fed FO compared to rats fed TO or SO. There were no significant differences in mineral balance. Rats fed SO or TO had a lower (P<0.005) TBARS than CO, KO, and MO, indicating that rats fed SO or TO had lower lipid peroxidation. Based on the study results, different sources of ω-3 PUFAs influenced bone differently. Rats fed FO or MO, rich in alpha-linolenic acid (ALA), promoted bone microarchitecture and rats fed TO, rich in docosahexaenoic acid (DHA), had increased bone mineralization. The study results suggest that rather than focusing on one source of ω-3 PUFAs, perhaps a variety of sources of ω-3 PUFAs should be consumed in order to improve bone health during the growth stage of rats.

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LIST OF ABBREVIATIONS

ω-3	Omega-3
ω-6	Omega-6
ALA	Alpha-linolenic acid
ALP	Alkaline phosphatase
ANOVA	Analysis of Variance
BMA	Bone mineral area
BMC	Bone mineral content
BMD	Bone mineral density
Ca	Calcium
CONN	Connectivity density
ddH_2O	Deionized distilled water
DEXA	Dual energy x-ray absorptiometry
DHA	Docosahexaenoic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
LA	Linoleic acid
P	Phosphorus
PGE_2	Prostaglandin E_2
PUFA	Polyunsaturated fatty acids
PYD	Pyridinoline
SMI	Structure model index
TBARS	Thiobarbituric acid reactive substances
TbN	Trabecular number
TbSp	Trabecular separation
TbTh	Trabecular thickness
TNF-α	Tumor necrosis factor alpha
μCT	Micro-computer tomography

1.0. Introduction

During childhood and adolescence, bone formation exceeds the rate of bone resorption resulting in bone acquisition. Peak bone mass (PBM) is the maximal amount of bone mass achieved during the growth stage (Anderson, 1996). After this stage, bone formation begins to decline while resorption continues at the same rate, resulting in bone loss (Martini, 2006). Bone loss occurs by demineralization and the breakdown of collagen in the bone matrix (Claassen et al., 1995b). Bone loss leading to decreased bone strength increases the risk of osteoporosis. In the absence of a cure for osteoporosis, emphasis is placed on the prevention of bone loss. Studies suggest that the onset of osteoporosis may be delayed and severity of osteoporosis may be lessened if bone modeling during the growth stage is optimized to achieve maximal PBM (Hansen, 1991; Matkovic, 1994). The lack of effective treatments for osteoporosis results in emphasis on prevention, such as dietary strategies to improve bone health during the growth stage. Dietary intervention to achieve maximal PBM and to prevent bone loss has focused on calcium (Ca) and factors influencing Ca balance.

Studies have reported that dietary fatty acid intake affect Ca balance (Cashman 2007, Claassen et al., 1995a; Hogstrom et al., 2007; Kruger et al., 1997; Kruger et al., 2005; Poulsen et al., 2007; Salari et al., 2008; Watkins et al., 2003; Weiss et al., 2005). Rats receiving a Ca adequate diet and provided fish oil supplements increased Ca transport across the basolateral membrane and decreased fecal Ca excretion (Claassen et al., 1995). EPA and DHA have been reported to increase Ca^{2+} ATPase activity, a membrane protein that transports Ca, that promotes Ca absorption and thereby, overall Ca balance. Omega-3 polyunsaturated fatty acids (ω-3 PUFAs) also affect various factors influencing osteoclasts and osteoblasts. The essential fatty acid linoleic acid (LA, 18:2 ω-6) can be metabolized to prostaglandin E_2 (PGE_2). PGE_2

stimulates bone formation at low concentrations, but inhibits at high concentrations (Mollard et al., 2005). High concentrations of PGE_2 also increased various cytokines observed to be active in the pathogenesis of osteoporosis (Salari et al., 2008).

The ω-3 PUFAs compete with ω-6 PUFAs for the same enzymes and therefore, may inhibit PGE_2 synthesis (Schwalfenberg 2006). The ω-3 PUFAs, alpha-linolenic acid (ALA, 18:3 ω-3), docosahexaenoic acid (DHA, 22:6 ω-3), and eicosapentaenoic acid (EPA, 20:5 ω-3) have been reported to reduce osteoclast activity, suppress bone resorption, and to increase bone mineral density (BMD) (Hogstrom et al., 2007; Griel, 2007; Weiler et al., 2002; Weiss et al., 2005). Various sources of ω-3 PUFA are commercially available as dietary supplements. However, sources of ω-3 PUFA may differ in fatty acid type, EPA:DHA ratio, and/or structural form. Therefore, the objective of this study was to determine the effect of feeding growing female rats different sources of ω-3 PUFAs on bone mineralization, microarchitecture, and strength. To our knowledge, this is the first study to compare the effect of different sources of ω-3 PUFAs on bone health.

2.0. Study Hypothesis and Objectives

Different sources of ω-3 PUFAs, due to their differing types of fatty acids, ratios of EPA and DHA and their structural form, will vary in their ability to affect mineral balance, bone mass, bone mineral content, and bone mineral density when consumed by young growing rats.

In order to test the hypothesis the study objectives were:

1. To determine the effects of feeding growing female rats ω-3 PUFAs from different sources on bone morphometry, bone mass, microarchitecture, and bone strength.
2. To determine potential mechanisms of ω-3 PUFAs from different sources on bone health in growing female rats.

3.0. Literature Review

3.1. Bone Biology

The skeletal system functions to support the body structurally, store minerals and lipids, produce blood cells, protect soft tissues, and act as levers to change direction and magnitude of the forces from skeletal muscles. Several segments make up the long bone (Figure 1). The diaphysis is the bone's shaft and the epiphyses are the proximal and distal ends of the bone (Martini, 2006). In a mature bone, the metaphyses are where the diaphysis joins the epiphyses. In a growing bone, the metaphyses include the epiphyseal plate. A tough sheath of dense irregular connective tissue called the periosteum covers the bone. Cartilage covers the bone where the bone forms a joint with another. The marrow cavity (i.e., medullary cavity) is the space within the diaphysis that contains bone marrow. The microarchitecture of the bone consists of epiphyses containing spongy or trabecular bone. Trabecular bone has a honeycombed structure and has a thin covering of cortical bone (Martini, 2006).

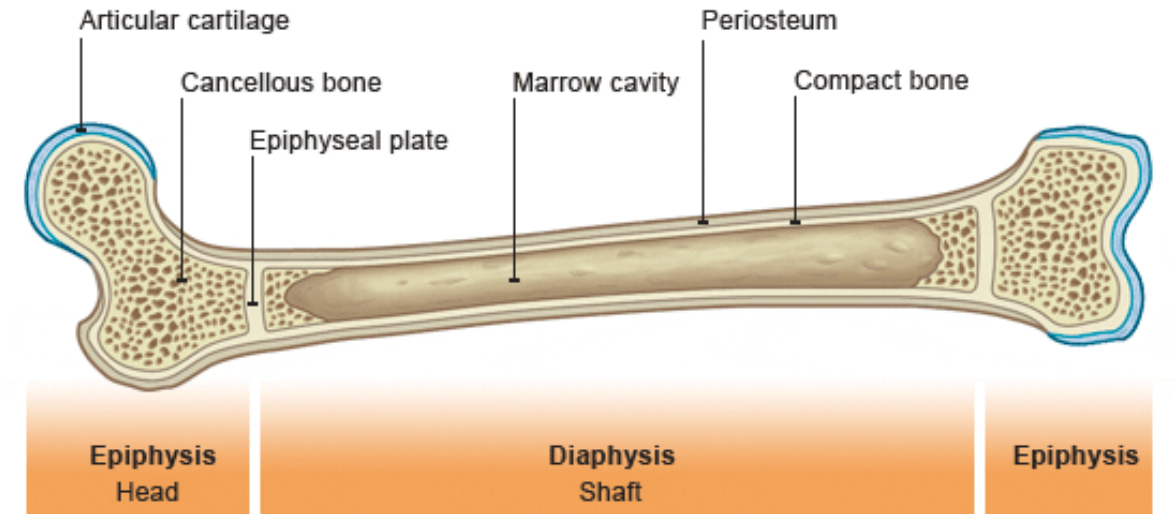


Figure 1: Structure of a bone (British Broadcasting Corporation <http://www.bbc.co.uk>)

In cortical bone, calcium phosphate [$Ca_3(PO_4)_2$] accounts for almost two-thirds of the weight of the bone (Martini, 2006). Calcium phosphate interacts with calcium hydroxide, [$Ca(OH)_2$] to form crystals of hydroxyapatite, [$Ca_{10}(PO_4)_6(OH)_2$]. Formation of hydroxyapatite crystals includes incorporation of other Ca salts and ions such as sodium, magnesium, and fluoride. The hard and brittle hydroxyapatite crystals are stored in the bone matrix and can withstand compression, but break when bent or twisted (Martini, 2006). In contrast, collagen fibers in the bone matrix are flexible and can tolerate twisting or bending, but not compression (Martini, 2006). The combination of the hydroxyapatite crystals and collagen fibers forms a strong bone that is somewhat flexible and highly resistant to fracture.

Demineralization and the breakdown of collagen in the bone matrix results in bone loss (Claassen et al., 1995b). Cortical mineralization can be determined by using dual energy x-ray absorptiometry (DEXA) to measure bone mineral density (BMD), bone mineral content (BMC) and bone mineral area (BMA). BMD represents a measurement of the bone mass per cm^2, BMC represents the mass of the bone, and BMA represents the area of bone occupied by minerals. Decreased BMD and BMC increase bone fragility and susceptibility to fracture (Cashman, 2007).

Bone architecture can be determined using micro-computed tomography (μCT). Trabecular bone volume to total tissue volume ratio (BV/TV) is the trabecular fraction of bone in the sample, trabecular number (TbN) is the number of trabeculae per mm of bone, trabecular thickness (TbTh) represents the mean diameter of the trabeculae, trabecular separation (TbSp) is the mean size of the intertrabecular spaces, connectivity density (CONN) is a quantified measurement of elasticity, and structure model index (SMI) characterizes the type of structure of the sample (rod-like, plate-like, or mixed structures). High values for BV/TV, TbN, TbTh and

CONN correlate with a strong bone. High values for TbSp indicate a weak bone. A bone having more rod-like structures is weaker than bone having more plate-like structures (Parkinson et al., 2009). For an ideal plate structure, SMI is close to 0 and for an ideal rod structure the value is 3.

3.2. Osteoporosis

As shown in Figure 2, loss of bone mineral and matrix (i.e. microarchitecture) is the major factor underlying osteoporotic fracture (Cashman, 2007).

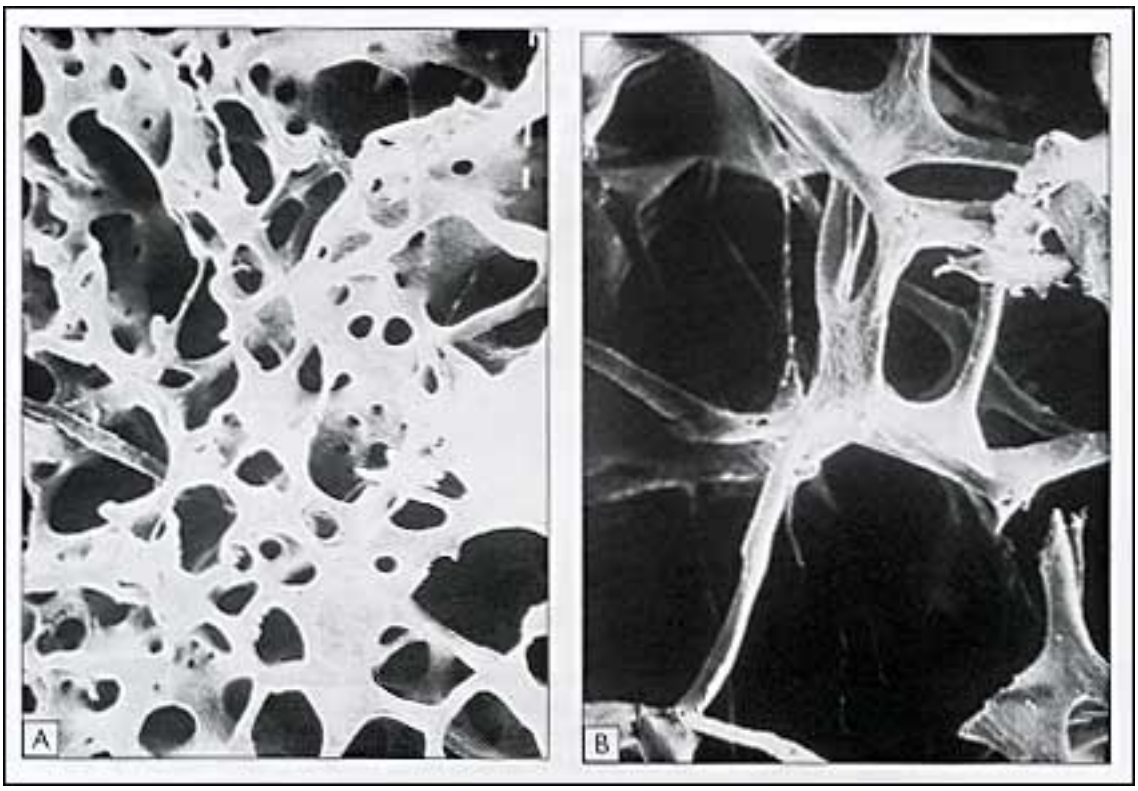


Figure 2: Scanning electron micrograph of a normal bone (Panel A) and a bone with osteoporosis (Panel B) <pdrhealth.com>

Fractures due to osteoporosis lower a patient's quality of life. According to the National Osteoporosis Foundation (2009), 1 in 5 individuals who were ambulatory before their hip fracture required long-term care afterward. Hip or vertebral fractures are also linked to an increased risk of death. An average of 24 percent of hip fracture patients, aged 50 and over, die

within a year following fracture. In the absence of a cure for bone loss leading to osteoporosis, maximizing PBM during growth by promoting bone formation is a major strategy for reducing the future risk of osteoporosis.

3.3. Bone Turnover and Growth

Cells account for 2% of the mass of a typical bone. There are 4 types of cells in the bone: osteocytes, osteoblasts, osteoprogenitor cells, and osteoclasts. Osteocytes maintain the protein and mineral content of the surrounding matrix and repair the bone when damaged (Martini, 2006). Osteoblasts produce new bone matrix, facilitate bone mineralization, and assist in increasing local concentrations of calcium phosphate and promoting the deposition of Ca salts in the bone matrix (Martini, 2006; Watkins et al., 2001a). Osteoprogenitor cells are stem cells that divide to produce daughter cells that differentiate into osteoblasts (Martini, 2006). These cells maintain osteoblast population and are important in the repair of a fracture. Osteoclasts are special macrophage-like cells (Poulsen, 2007) that remove bone matrix (Martini, 2006) in a process called resorption. Resorption is important in the regulation of blood Ca and P concentrations. Ca and P are mobilized from bone stores when blood Ca and P concentrations are low.

The process of resorption and formation of new bone known as bone turnover is continuous. Bone turnover begins with the recruitment of osteoclasts from bone marrow precursors (Watts, 1999). Osteoclasts secrete proteolytic enzymes and acids to dissolve the matrix and release the stored minerals (Martini, 2006). After removal of old bone, osteoblasts differentiate from connective-tissue precursors and release proteins to form new bone matrix. Bone formation is coupled to bone resorption, but do not always occur at the same rate. Growth

of the bone occurs when bone formation exceeds bone resorption. In rats, bone continues to grow until age 6 months after which bone growth slows (Martin, 2003). In humans, after the age of 35-40, bone resorption exceeds bone formation resulting in a net loss of bone (Watts, 1999).

3.4. Bone Turnover Markers

There are three categories of bone turnover markers: enzymes/proteins that are secreted by cells involved in remodeling process, breakdown products generated in the resorption of old bone, and byproducts produced during the synthesis of new bone (Watts, 1999). A collagen breakdown product commonly used as an indicator of osteoclast activity (i.e. bone resorption) is pyridinoline (PYD) cross-links. A high level of serum PYD indicates that bone resorption is occurring within the bone.

Serum osteocalcin is commonly used as an indicator of bone formation (osteoblast activity). Osteocalcin is a bone-specific protein released into the blood during new matrix formation (Poulsen et al., 2007). Osteoblasts are rich in alkaline phosphatase, however the function of this enzyme is unknown (Watts, 1999). Osteoblast as well as osteoclast activity regulate bone mass (Poulsen et al., 2007). Bones weaken when osteoclasts remove bone faster than osteoblasts deposit bone (Martini, 2006). A low bone mass result in decreased bone strength, and decreased bone strength (bone fragility) is defined as susceptibility to fracture (Turner, 2002).

One issue with turnover markers is that it only provides information at a single time point. To prevent this, multiple measurements must be made over a time period in order to understand the true value of the marker. However, the amount of blood required to do multiple

testing is greater than the amount found in a rat. For that reason, rat studies usually only do a single time point measurement of turnover markers.

3.5. Bone Biomechanics

Bone fragility is defined by biomechanical parameters. These parameters include: strength, brittleness, work to failure, and stiffness (Turner, 2002). Shown in Figure 3 is a force-displacement curve generated by strength testing used to calculate bone biomechanical parameters.

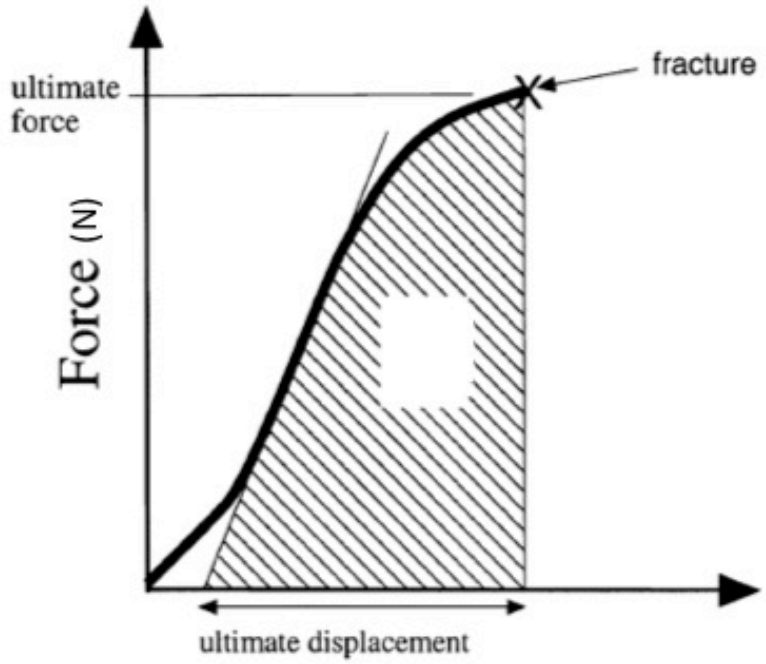


Figure 3: Force-displacement curve resulting from a biomechanical test of a bone specimen. Adapted from Turner 2002.

Peak force is defined as the highest point of the curve. Ultimate stiffness is the slope of the curve. Bending energy failure is defined as the area under the curve and brittleness is

estimated from the reciprocal of the curve (Turner, 2002). Ultimate bending stress is a normalized calculated force that takes into account the size of the bone and Young's Modulus is normalized stiffness that also takes into account bone size (Yuan et al., 1992).

3.6. Peak Bone Mass

PBM is defined as the highest level of bone mass achieved during the growth stage (Hind et al., 2007). PBM is achieved a few years after the fusion of the epiphyses (Heaney, 2000). It is important to maximize PBM during the growth stage because during this stage, increased bone formation results in increased bone accrual, size, and bone strength (Hind et al., 2007). After this stage, around age 35 to 40 years old in humans, bone resorption exceeds bone formation resulting in steady bone loss. Females are at increased risk of osteoporosis due to their lower PBM compared to males (Nieves, 2005). Therefore, female rats were used in this study. Low PBM is a recognized "risk factor" for osteoporosis later in life (Hawker et al., 2002). To prevent future risk of osteoporosis it is important to identify modifiable factors that may improve PBM during this critical stage of bone acquisition. Nutrition is an important modifiable factor that may influence PBM. Recently, there has been increasing evidence that dietary fatty acids and their eicosanoid derivatives affect bone metabolism. The next section reviews ω-3 PUFAs and their role in bone health.

3.7. Omega-3 Polyunsaturated Fatty Acids

An essential nutrient is one that must be obtained from the diet because the body cannot produce it in adequate amounts. The essential fatty acid linoleic acid (LA, 18:2 ω-6), when consumed in sufficient quantities may be converted to the long-chain PUFA, arachidonic acid

(AA, 20:4 ω-6) by endogenous desaturase and elongase enzymes. Eicosanoids can be synthesized from AA by the cyclooxygenase (COX) enzymes (Figure 4). Eicosanoids are biologically active compounds that are involved in modulating the intensity and duration of inflammatory responses (Calder, 2002). AA produces the n-2 series prostaglandin (PG), one of which is PGE_2. PGE_2 regulates inflammatory cytokines such as: TNF-α, IL-1, and IL-6. Therefore, overproduction of AA-derived eicosanoids may place the body in a pro-inflammatory state (Calder, 2002; Watkins et al., 2001a). The dramatic rise in LA intake over the last 50 years from the widespread use of vegetable oil such as corn oil, may lead to a pro-inflammatory state (Fritsche, 2008). An acute inflammatory response is useful, for it isolates the damaged area, mobilize effector cells to the site of injury, and promote healing (Kimball, 2010)

The ω-3 essential fatty acid, alpha-linolenic acid (ALA, 18:3 ω-3) when consumed in sufficient quantities may be converted to long chain PUFA, eicosapentaenoic acid (EPA, 20:5 ω-3). EPA may then be converted to docosahexaenoic acid (DHA, 22:6 ω-3). ALA is converted to EPA using the same desaturase and elongase enzymes used to convert LA to AA. Therefore, LA and ALA may competitively inhibit one another. Competitive inhibition of LA by ALA reduces AA. This in turn, may result in a reduction of pro-inflammatory PGE_2.

Marine oils provide a rich source of the long-chain ω-3 PUFAs, EPA and DHA. This is of health significance because EPA competes with AA for the COX enzyme regulating eicosanoid synthesis (Shane et al., 2002). Competitive inhibition of AA by EPA also reduces n-2 series pro-inflammatory eicosanoids and increases synthesis of the less inflammatory n-3 series eicosanoids (Figure 4). The richest source of ALA is flaxseed (Cunnane, 1995). However conversion of ALA to EPA (0.2%) and DHA (0.5%) is inefficient (Ratnayake et al., 2009). Therefore, consuming foods rich in EPA and DHA is recommended by the American Heart

Association, American Dietetics Association, and Council for Responsible Nutrition (Ismail, 2010). Consumption of EPA and DHA from fish or fish oil has been shown to partially replace the ω-3 PUFA that are in the phospholipid bilayer in white blood cell (Salari et al., 2008; Simopoulos, 1991).

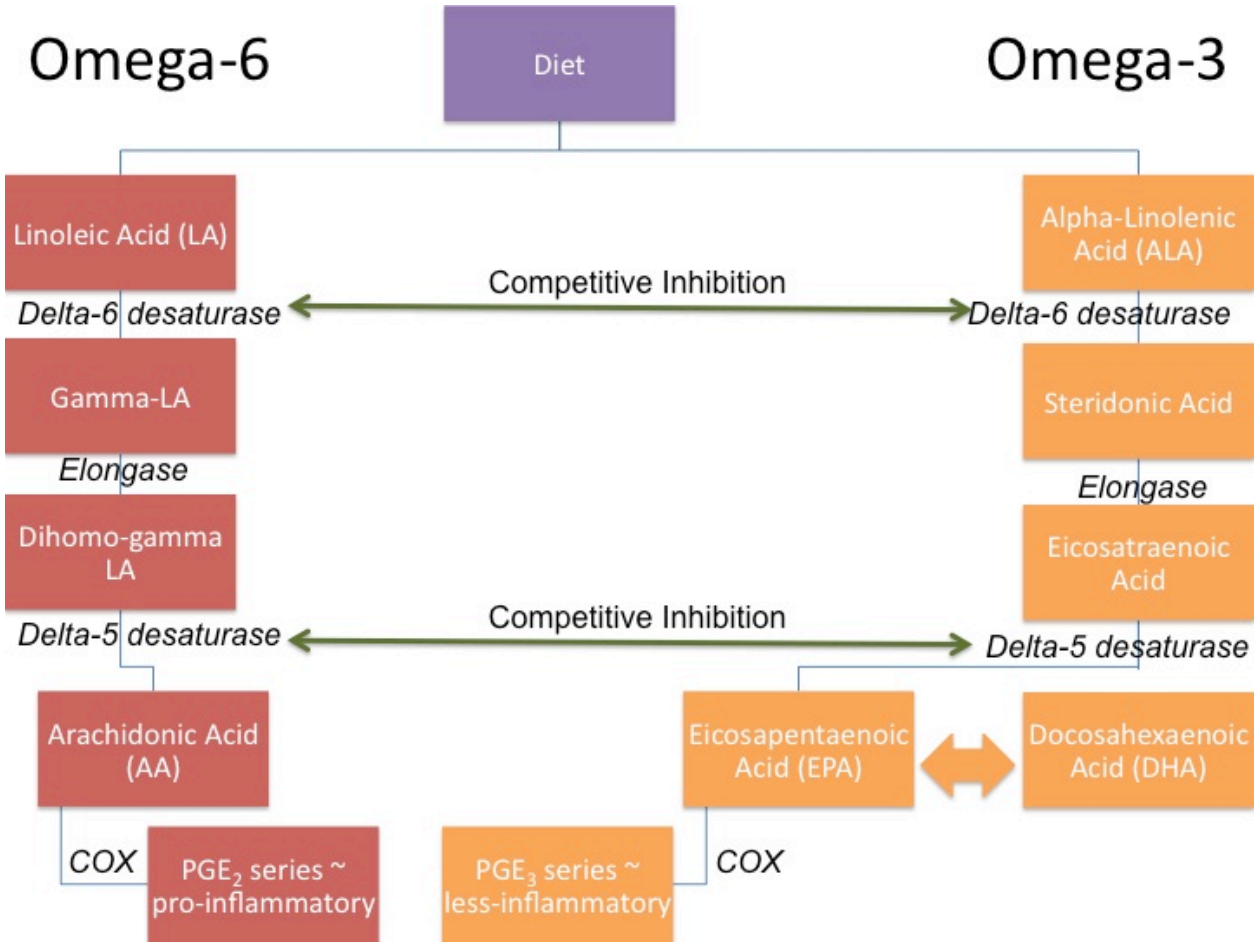


Figure 4: Polyunsaturated fatty acid metabolism

Marine oils provide a rich source of the long-chain ω-3 PUFAs, EPA and DHA. A popular commercial fish oil supplement is menhaden oil. Salmon and tuna are commonly eaten fish in the Western diet. Krill is another seafood source of ω-3 PUFAs. In krill oil, fatty acids are associated mainly with phospholipids; whereas, fatty acids in fish oil are associated with triglycerides (Bottino, 1975; Gigliotti et al., 2010). Phospholipids have been suggested to be

better absorbed than triglycerides (Amate et al., 2001). Therefore, in this study, flaxseed oil, krill oil, and three fish oils (salmon, tuna, and menhaden) were the different sources of ω-3 PUFAs used.

It has been suggested by the American Heart Association that ω-3 PUFA consumption should be increased. Currently, the ratio of ω-6:ω-3 is at ~10:1 in a typical Western diet (Kris-Etherton et al., 2003). However, Simopoulous (2008) has suggested that the ω-6:ω-3 ratio should be closer to 2:1. Cell membrane changes due to the ratio of ω-6:ω-3 PUFA may lead to changes in tissue function, such as production of cytokines (Simopoulos, 2002). Calder (2002) suggested that increasing ω-3 PUFAs in membranes might decrease inflammation by reducing the production of pro-inflammatory eicosanoids (i.e. PGE_2) and cytokines (i.e. TNF-α). PGE_2 is involved in inflammation for it induces fever, increase vascular permeability and vasodilatation, and enhancing pain and edema (Calder, 2002). It also suppresses lymphocyte proliferation and inhibits production of IL-2, thus, in these respects PGE_2 is immunosuppressive (Calder, 2002). It is important to note that an excess of ω-3 PUFAs will decrease coagulation in the blood, which could result in hemorrhaging. Therefore, it is important to establish safe levels of ω-3 PUFAs. The next section reviews the effect of ω-3 PUFA in bone tissue.

3.8. Omega-3 Polyunsaturated Fatty Acids and Bone Health

While is has been well established that ω-3 PUFAs are heart healthy, its effects on bone are not as well known. Claassen et al. (1995b) investigated whether the ω-3 PUFA EPA, provided as fish oil, and ω-6 PUFAs gamma-LA (γ –LA) provided as primrose oil, influenced bone status in growing (age 21 days) male Sprague-Dawley rats. Rats were fed a diet supplemented with different ratios of ω-3 PUFA (as fish oil) and ω-6 PUFA (as evening

primrose oil) for 3 months. Rats fed diets of 1:3 γ-LA:EPA had reduced bone matrix collagen degradation ($P<0.05$) compared to the control rats fed 3:1 of LA:ALA as measured by bone resorption biomarkers. Results suggested a higher ω-3 PUFA ratio may be beneficial to bone. In a human study of adolescent white males (average age 16.7 years), a higher ratio ω-6:ω-3 PUFA intake showed a negative correlation to spine BMD (r=-0.26, P=0.02); whereas, serum DHA and ω-3 PUFA concentrations were positively correlated to spine (r=0.30, P=0.008) and total body (r=0.32, P=0.004) BMD (Hogstrom, 2007). The results suggested consumption of ω-3 PUFA, especially DHA, increased bone mineralization during growth.

Dietary fatty acids affect the bone by various mechanism of action. In a human study, Griel et al. (2007) fed 20 male and 3 female subjects (average age of 49 years) plant-derived ω-3 PUFAs. The diets consisted of a typical American diet (control, 9:1 ω-6:ω-3) or high ω-3 diets consisting of ratios 3.5:1 and 1.6:1. Total fat content was ~35% of energy. Sources of the oil came from walnut and flaxseed oil, which are rich in the ω-3 PUFA, ALA. Subjects fed high ω-3 PUFAs compared to the typical American diet high in ω-6 fatty acids, had reduced osteoclast activity indicated by reduced serum N-telopeptide concentrations and maintained osteoblast activity indicated by no change in bone-specific alkaline phosphatase. The results showed the high ω-3 PUFA diet prevented bone loss by decreasing bone resorption while maintaining bone formation. However, the study results did not distinguish whether the effects were due to ALA or the conversion of ALA to EPA.

Indirectly, dietary fats may affect bone by altering Ca balance. Kruger et al. (2005) reported young (age 5 weeks) male Sprague-Dawley rats fed 5% tuna oil g/kg diet had significantly higher Ca absorption and decreased urinary Ca compared to the rats fed evening primrose oil. The authors suggested that cell membrane saturation reduced Ca absorption and

decreased cell membrane saturation due to ω-3 PUFA consumption resulting in increased Ca absorption and decreased urinary Ca loss. Further, the authors suggested that in addition to absorption and excretion, essential fatty acids LA or ALA may affect Ca retention. Claassen et al. (1995a) fed growing (age 21 days) male Sprague-Dawley rats different ratios of γ-LA:EPA. Ratio of 3:1 of γ-LA:EPA decreased Ca excretion and led to rats with higher (P<0.05) bone Ca content compared to the control ratio of 3:1 of LA:ALA.

Another potential mechanism whereby ω-3 PUFAs may influence bone is by altering the synthesis of PG. Watkins et al. (2000) compared the effect of different dietary ratios of ω-6:ω-3 PUFA on PGE_2 production and bone. Weanling (age 21 d) male Harlan Sprague-Dawley rats were fed a standard purified 7% soybean oil g/kg diet in American Institute of Nutrition 93G diet (AIN-93G, Harlan Teklad, WI) (control diet), or AIN-93G with 7% test oil g/kg diet containing different proportions (ω-6:ω-3 ratio of 9:1, 4:1, 1:1, and 1:2.3; wt/wt) of safflower oil, a rich source of ω -6 PUFAs, and menhaden oil, a rich source of ω-3 PUFAs, EPA and DHA. Rats fed higher amounts of menhaden oil for 42 days had lower production of PGE_2 in the liver (P=0.0002), tibia (P=0.02) and femur (P=0.006). Serum bone-specific alkaline phosphatase, an indicator of osteoblast activity, was also higher (P=0.04) in rats consuming a greater amount of ω-3 PUFA. Regression analysis showed a negative correlation between high ω-6: ω-3 ratio and bone formation as indicated by bone-specific alkaline phosphatase (r^2=0.34, P=0.01). Also, a negative correlation was shown between PGE_2 and bone formation (r^2=0.22, P=0.05). The results indicated increasing ω-3 PUFA intake promoted bone formation by increasing osteoblast activity by bone-specific alkaline phosphatase and reducing the PGE_2 associated with high ω-6 PUFA consumption.

Another possible mechanism of action of ω-3 PUFAs on bone is the potential of PG to alter cytokine production. Inflammatory cytokines such as TNF-α are known to regulate osteoblastic cells (Walsh et al., 2006). TNF-α inhibits the differentiation of osteoblasts and inhibits collagen synthesis by osteoblasts (Walsh et al., 2006). Ferrucci et al. (2006) reported a diet high in ω-3 PUFA lowered AA derived cytokines such as IL-6, TNF-α, and C-reactive protein. In summary, dietary fats potentially affect the bone by various mechanisms that include alteration in Ca absorption and urinary Ca loss, PG synthesis, and cytokine synthesis (Salari et al., 2008).

4.0. Materials and Methods

4.1. Animals and Diets

The Institutional Animal Care and Use committee at West Virginia University approved the protocol for this study. All animal procedures used in this study conformed to the National Research Council (NRC) Guide for the Care and Use of Laboratory Animals. Growing (age 28 days) female Sprague-Dawley rats (n=60) were obtained from Hilltop Laboratory (Scottdale, PA). Rats were individually housed in metabolic cages under conditions of controlled temperature (22 ± 2^0C) and a 12-h light:dark cycle. During the 7-day acclimation, all animals were given *ad libitum* access to deionized distilled water (ddH_2O) and the American Institute of Nutrition 93G diet (AIN-93G, Harlan Teklad, WI). The AIN-93G was fed because it is a purified diet that provides precise levels of minerals, and meets the National Research Council (1995) nutrient recommendation for growing rats.

After 7 days acclimation, the rats were randomly assigned (n=10 rats/group) to a high fat (12% by weight lipid diet, ~27% by kcal) diet consisting of: 1) corn oil (CO), 2) flaxseed oil (FO), 3) krill oil (KO), 4) menhaden oil (MO), 5) salmon oil (SO), or 6) tuna oil (TO). CO was added to MO and KO in order to meet the NRC recommendation for the essential ω-6 PUFA, LA. Treatment diet was formulated based on the AIN-93G formulation using 12% instead of 7% lipid g/kg diet. A high fat diet was used to imitate the typical Western human diet. All diets were adjusted to have a similar fat content, to be isocaloric, and to contain 357 mg/kg diet of calcium carbonate and 196 mg/kg diet of potassium phosphate. Diet composition is shown in Table 1. Fatty acid composition of dietary oils is shown in Table 2. CO was chosen for its prevalence in the typical Western diet and for its high ω-6:ω-3 ratio. FO was chosen because it is high in the ω-3 PUFA, ALA and does not contain EPA or DHA. KO was chosen since its

fatty acids are mainly in phospholipid form instead of triglyceride form (Gigliotti et al., 2010). KO and MO have the same EPA:DHA ratio, however MO is in the triglyceride form. MO was chosen because it is a common source used in studies. SO and TO are common sources of fish oil supplements and are commonly eaten fish. TO has a lower EPA:DHA ratio than SO. CO, FO, MO, SO, and TO were kindly provided by J. Edwards International Inc. (Quincy, MA). KO was obtained from Enzymotec Ltd. (Morristown, NJ). Oils were analyzed for fatty acid content by gas chromatography. Lipid classes were separated and quantified by thin layer chromatography (TLC) according to Gigliotti et al. (2011). Rats were fed 14.75 ± 0.75 g diet/day of their assigned diet and provided *ad libitum* access to ddH_2O throughout the 8 week feeding study. Food intake and spillage was recorded and fresh diet was provided daily. Water consumption and body weights were measured weekly.

Table 1: Ingredient Composition of the Treatment Diets

Ingredients (g/kg diet)	CO	FO	KO	MO	SO	TO
Test oil	0.0	120.0	118.0	118.0	120.0	120.0
Corn oil	120.0	0.0	2.0	2.0	0.0	0.0
Casein	200.0	200.0	200.0	200.0	200.0	200.0
L-cysteine	3.0	3.0	3.0	3.0	3.0	3.0
Corn starch	347.5	347.5	347.5	347.5	347.5	347.5
Maltodextrin	132.0	132.0	132.0	132.0	132.0	132.0
Sucrose	100.0	100.0	100.0	100.0	100.0	100.0
Cellulose	50.0	50.0	50.0	50.0	50.0	50.0
Vitamin mix	10.0	10.0	10.0	10.0	10.0	10.0
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
Mineral mix[1]	35.0	35.0	35.0	35.0	35.0	35.0
Calcium Carbonate	0.357	0.357	0.357	0.357	0.357	0.357
Potassium Phosphate (monobasic)	0.196	0.196	0.196	0.196	0.196	0.196

[1] Ingredients of the AIN-93G Mineral Mix (g/kg): Calcium Carbonate = 357, Potassium Phosphate = 196, Potassium Citrate = 70.78, Sodium Chloride = 74, Potassium Sulfate = 46.6, Magnesium Oxide = 24.3, Ferric Citrate = 6.06, Zinc Carbonate = 1.65, Manganous Carbonate = 0.63, Cupric Carbonate =0.31, Potassium Iodate = 0.01, Sodium Selenate = 0.0103, Ammonium Paramolybdate = 0.008, Sodium Meta-Silicate = 1.45, Chromium Potassium Sulfite = 0.275, Lithium Chloride = 0.0174, Boric Acid = 0.0815, Sodium Fluoride = 0.0635, Nickel Carbonate Hydroxide = 0.0318, Ammonium Meta-Vanadate = 0.0066, Sucrose = 220.716. Abbreviations are: CO=corn oil, FO=flaxseed oil, KO=krill oil, MO=menhaden oil, SO=salmon oil, TO=tuna oil.

Table 2: The ω-6 and ω-3 Polyunsaturated Fatty Acid Composition of Dietary Oils[2]

Fatty Acid[1]	CO	FO	KO	MO	SO	TO
ω-6 PUFAs						
Linoleic acid (LA, 18:2 ω-6)	57.8	15.5	1.9	0.7	4.0	1.7
Arachidonic acid (AA, 20:4 ω-6)	0	0	0	0	0	1.9
ω-3 PUFAs						
Alpha-linolenic acid (ALA, 18:3 ω-3)	1.0	55.9	1.3	3.1	1.4	0.7
Eicosapentaenoic acid (EPA, 20:5 ω-3)	0	0	20.3	9.0	14.4	8.6
Docosahexaenoic acid (DHA, 22:6 ω-3)	0	0	5.9	3.3	3.5	24.8
EPA:DHA	n/a	n/a	3:1	3:1	4:1	1:3
ω -6: ω -3	60:1	1:4	1:14	1:23	1:5	1:9

[2] Results are given as percent of total fatty acid. Abbreviations are CO=corn oil, FO=flaxseed oil, KO=krill oil, MO=menhaden oil, SO=salmon oil, TO=tuna oil, PUFA=polyunsaturated fatty acids

4.2. Bone Morphometry

At the end of the 8-week feeding study, the rats were food-deprived overnight. Rats were euthanized by carbon dioxide inhalation. Both the left and right femurs and tibiae bones were collected by disarticulating the hip joint. The long bones were cleaned of all soft tissue. Each bone was wrapped in ddH_2O-soaked gauze and stored at -20^0C until analyzed.

For analysis, each bone was brought to room temperature. Morphometry measurements of bone length, width, and depth were measured using a vernier caliper (Bel-Art Products, Pequannock, NJ). Length was measured from medial condyle to greater trochanter. Diameter was measured at mid-section of shaft from lateral to medial end (i.e. diaphysis). Bones were dried at 110^0C for 48 h then were weighed using an analytical balance (A-200DS, Fisher Scientific).

4.3. Bone Mineral, Calcium, and Phosphorus Content

The left long bones were packed on dry ice and shipped overnight to Dr. Brenda Smith at Oklahoma State University to determine BMA, BMC, and BMD of the whole tibia and femur by dual-energy x-ray absorptiometry (DEXA) (Hologic QDR 4500-A Elite). BMA, BMC, and BMD scans were evaluated using the Regional High Resolution software package (Hologic Waltham, MA). To determine total bone ash, femurs and tibiae were dried for 48 h at 110^0C then placed in a muffle furnace (model CP18210, Thermolyne, Dubuque, IA) at 600^0C for 24 h. Total ash was determined by weighing the bone ash. To determine Ca and P, ashed bones were crushed and acidified in 2 mL of 6M nitric acid. The acidified samples were neutralized in 5 ml ddH_2O and filtered through Whatman no. 1 paper. Samples were diluted to a final concentration

of 1:500 ddH_2O. Ca and P concentrations in samples were determined by inductively coupled plasma optical emission spectrophotometry (ICP, model P400, Perkin Elmer, Shelton, CN).

4.4. Bone Microarchitecture

Trabecular and cortical bone architecture were determined in the left bones using micro-computer tomography (μCT) (MicroCT40, SCANCO Medical, Switzerland). The distal femur metaphysis and proximal tibial metaphysis were scanned and 200 images (~16 μm/slice or 3.2 mm) were analyzed by semi-automatically placing contours beginning 25 slices (400 μm) away from the growth plate and includes only secondary spongiosa within the volume of interest (VOI). Cortical bone architecture of the femur and tibia mid-diaphysis were evaluated on 32 slices (512 μm). Cortical indices included the medullary area and cortical thickness, area, and porosity. All scans were performed utilizing a 1024 x 1024 matrix resulting in an isotropic voxel resolution of 22 μm^3. An integration time of 70 milliseconds per projection was used with a rotational step of 0.36 degrees resulting in total acquisition time of approximately 150 minutes/sample. The VOI was assessed for structural parameters including trabecular bone volume per unit of total volume (BV/TV), trabecular number (TbN), trabecular thickness (TbTh), trabecular separation (TbSp), structure model index (SMI), and connectivity density (CONN).

4.5. Bone Biomechanics

Bone strength indices were determined using a TA.XT2i Texture Analyzer (Texture Technologies, Scarsdale, NY) outfitted with a three-point bending apparatus. Femora and tibiae were placed on supports. Span between the supports was 1 cm for both the femora and tibia.

The bone was bent until broken by lowering a centrally placed blade (1 mm width) at a constant crosshead speed (0.1 mm/sec) and load cell of 250kg. A force-deformation curve (Figure 3) was used to determine mechanical properties.

Peak force (N): the maximum force obtained during the bending procedure resulting in the initiation of the bone breaking.

Ultimate stiffness (N/s): the slope of the time-force deformation curve.

Ultimate bending stress (UBS, N/mm^2): a calculation of force that takes into consideration the size of the bone calculated as:

$$UBS = \frac{8 \times \text{peak force} \times L \times a_1}{\text{Л}((a_1^3 a_2)-(b_1^3 b_2))}$$

Where L is the distance between the supporting points; a_1= the outer diameter in the direction of the load, a_2= the outer diameter at right angles to a_1, b_1=the inner diameter in the load direction and b_2=the inner diameter at right angles to b_1.

Young's Modulus (N/mm^2): a calculation of stiffness that takes into consideration the size of the bone calculated as:

$$\text{Young's Modulus} = \frac{4 \times L^3 \times \text{ultimate stiffness (slope)}}{3 \times \text{Л}((a_1^3 a_2)-(b_1^3 b_2))}$$

Where L=the distance between the two edges in the three-point bending, a_1= the outer diameter in the direction of the load, a_2 = the outer diameter at right angles to a_1, b_1=the inner diameter in the load direction and b_2 = the inner diameter at right angles to b_1.

Bending Energy Failure (N.s): the area under the time-force deformation curve.

4.6. Mineral Balance

Ca and P balance was determined according to Tsanzi et al. (2008). Briefly, rats were housed individually in metabolic cages to determine food and water intake and to collect feces and urine. Treatment diets' Ca and P intake were determined by ICP (model P400) and Ca and P intake calculated by multiplying by their food intake. Ca intake was calculated as food intake (g) x 0.5% Ca. P intake was calculated by food intake (g) x 0.3% P.

Feces and urine were collected at baseline and during the final week of the 8-week feeding study. Ascorbic acid (0.1%) was added to the urine collection tubes as a preservative and 1 mL mineral oil to prevent evaporation. Collected urine samples were centrifuged at 1,500 *g* for 10 minutes at 4^0C. Following centrifugation, urine samples were aliquoted into fresh tubes and kept at -20^0C until assayed. Urinary concentration of creatinine was measured using a commercially available enzyme immunoassay (EIA) kit (Quidel Corp, CA). Urine samples were corrected for urinary volume. No significant differences were found in the creatinine values among the treatment groups; therefore, it was unnecessary to correct the urinary Ca values by dividing by the creatinine values.

Collected fecal samples (200 mg/rat) were freeze-dried for 48 h and then ashed in a muffle furnace at 600^0C for 18-24 h. Ashed fecal samples were acidified with 2 mL of 6M nitric

acid. The acidified samples were neutralized in 5 ml ddH_2O and filtered through Whatman no. 1 paper and diluted to a final concentration of 1:50. Fecal and urinary Ca and P concentrations were determined by ICP (model P400).

Ca apparent absorption was calculated as [(Ca intake – fecal Ca excretion)/Ca intake] x 100. Calcium retention was determined by calculating [Total Ca intake – (fecal Ca excretion + urinary Ca excretion)]. P absorption and retention was calculated using the same formulas.

4.7. Bone Turnover Markers

Serum was obtained at time after euthanasia and then centrifuging trunk blood samples at 1,500 *g* for 10 minutes at 4^0C. Serum samples were stored at -80^0C until assayed. Serum osteocalcin and alkaline phosphatase were measured as indicators of osteoblast activity (bone formation). Serum alkaline phosphatase was determined by veterinary test rotor colorimetric assay (Vet-16) and measured using a Hemagen Analyst automated spectrophotometer (Hemagen Diagnostics Inc, Columbia, MD). Serum osteocalcin was determined as an indicator of osteoblastic activity using a commercially available rat specific enzyme-linked immunosorbent assay (ELISA) (Biomedical Technologies, Stoughton, MA). Serum PYD was determined as an indicator of osteoclast activity using a commercially available ELISA kit (Quidel Corporation, CA). Optical density was measured at 450 nm using a Spectramaz Plus microplate reader (Molecular Devices, Sunnyvale, CA).

4.8. Biochemical Measurements

Serum Ca and P were determined by Vet-16 rotor colorimetric assay and measured using a Hemagen Analyst automated spectrophotometer (Hemagen Diagnostics Inc, Columbia, MD).

To measure the short lived molecule, PGE_2, in the urine, its metabolite 13, 14-dihydro-15-keto PGE_2 was determined using a commercially available EIA assay kit (Cayman Chemicals, Ann Arbor, MI). Serum TNF-α was determined using a commercially available EIA assay kit (Cayman Chemicals, Ann Arbor, MI). Absorbance for both PGE_2 and TNF-α was read at 405 nm using a Spectramax Plus microplate reader (Molecular Devices, CA).

To determine lipid peroxidation, thiobarbituric acid reactive substances (TBARS) were measured using a commercially available colorimetric EIA kit (Cayman Chemical, Ann Arbor, MI). Absorbance was read at 540 nm using a Spectramax Plus microplate reader (Molecular Devices, CA). Total antioxidant capacity was measured using a commercially available antioxidant assay colorimetric EIA kit (Cayman Chemical, Ann Arbor, MI). Absorbance was read at 750 nm using a Spectramax Plus microplate reader (Molecular Devices, CA).

4.9. Statistical Analysis

Results are expressed as means ± standard error of the mean (SEM). A one-way analysis of variance (ANOVA) was used to determine differences among the treatment groups. Post-hoc multiple comparison tests were performed using Tukey's test (parametric) with treatment differences considered significant at $P<0.05$. Kruskal-Wallis on Ranks (non-parametric) was used. All statistical analyses were done using the statistical software SigmaStat 3.1 (Systat Software Inc, San Jose, CA).

5.0. Results

5.1. Food Intake and Body Weights

No differences were observed in total food intake, weight gain, or final body weight among the treatment groups (Table 3)

5.2. Bone Morphometry

In the femur, depth measured at diaphysis was smaller (P=0.03) in KO-fed rats compared to FO-fed rats. There were no significant differences in femoral length, width, or dry weight among the different treatment groups (Table 4).

In the tibiae, rats fed TO had longer bones (P<0.001) than rats fed CO, FO, KO, or MO. Rats fed SO and TO had heavier (P<0.001) tibiae dry weight compared to KO or CO-fed rats. FO (P=0.04) and MO-fed rats (P=0.006) had heavier tibiae dry weight compared to CO-fed rats. There were no significant differences in depth or width in the tibiae among the different treatment groups (Table 4).

5.3. Bone Mineralization

In the femur, there were no significant differences in BMA, BMC, BMD, bone ash, Ca, or P content among the treatment groups (Table 5).

In the tibia, BMA of TO or SO-fed rats was higher compared to rats fed CO (P<0.001), KO (P=0.01), or MO (P=0.03). Rats fed TO or SO had higher BMC (P<0.001) than CO-fed rats. BMD in was higher (P=0.006) in TO-fed rats compared to CO-fed rats (Table 5). Ash

weight was higher (P<0.001) in rats fed TO, SO, MO, or FO compared to rats fed KO or CO. There was no significant difference in tibia Ca or P content (Table 5).

5.4. Bone Microarchitecture

In the femur, feeding different sources of ω-3 PUFAs resulted in significant differences in trabecular μCT measurements of BV/TV, TbN, TbSp, and CONN but had no effects on SMI or TbTh. Rats fed FO or MO had greater BV/TV (P=0.002), TbN (P=0.003), and CONN (P=0.002) and lower TbSp (P=0.003) than CO-fed rats (Table 6).

In the tibia, feeding different sources of ω-3 PUFAs resulted in significant differences in trabecular μCT measurements of BV/TV, TbN, ThTh, TbSp, CONN, and SMI. FO-fed rats had higher (P=0.007) BV/TV than rats fed CO or SO. Rats fed FO or MO had a higher TbN (P=0.003) than rats fed CO. Rats fed FO had a higher (P=0.04) TbTh than SO-fed rats. Rats fed FO or MO had higher (P=0.013) CONN compared to rats fed CO. FO, KO, MO, or TO-fed rats had lower (P<0.001) TbSp compared to rats fed CO (Table 6). FO-fed rats had a lower SMI compared to CO-fed rats (P=0.04) and SO-fed rats (P=0.02).

In the femur, cortical μCT resulted in no significant differences in cortical area, CtTh, and porosity among the treatment groups. Cortical femur BV/TV was lower (P=0.02) in SO-fed rats compared to CO-fed rats. In the cortical tibiae, there were no significant differences in BV/TV, CtTh, cortical area, and porosity among the treatment groups (Table 7).

5.5. Bone Biomechanical Strength

There was no significant difference in femur or tibia biomechanical strength measures of peak force, ultimate stiffness, ultimate bending stress, Young's Modulus, or bending energy failure energy (Table 8).

5.6. Mineral Balance

There were no significant differences in baseline Ca intake, urinary Ca excretion, nor Ca retention. There were no significant differences in final Ca urine, fecal excretion, apparent absorption or retention (Table 9). Rats fed SO had a higher fecal Ca excretion compared to FO ($P<0.001$), KO ($P=0.02$), or MO ($P<0.001$). Rats fed TO had a higher fecal Ca excretion compared to FO ($P<0.001$), KO ($P=0.005$), or MO ($P<0.001$). Rats fed FO and MO had a lower ($P=0.02$) fecal Ca excretion compared to CO. Baseline apparent absorption of Ca was higher in FO compared to CO ($P=0.03$), SO ($P<0.001$), or TO ($P<0.001$). It was also higher in MO compared to CO ($P=0.02$), SO ($P<0.001$), or TO ($P<0.001$) (Table 9).

At baseline, rats fed KO had a higher ($P<0.001$) P intake compared to CO, FO, MO, SO, or TO (Table 10). Rats fed SO also had a higher P intake at baseline compared to CO ($P=0.002$), FO ($P<0.001$), MO ($P=0.02$), or TO ($P=0.02$). At final, rats fed KO had a higher ($P<0.001$) P intake compared to CO, FO, MO, SO, or TO. Rats fed SO had a higher P intake at final compared to CO ($P=0.006$), FO ($P=0.001$), or MO ($P=0.005$). Rats fed KO, SO, or TO had a higher ($P<0.001$) fecal P excretion at baseline compared to FO or MO. Rats fed KO had a higher final P fecal excretion than rats fed FO ($P<0.001$) or MO ($P<0.001$) or TO ($P=0.03$) (Table 10). At baseline, rats fed FO had a higher urinary P excretion compared to MO ($P=0.03$), SO ($P<0.001$), or TO ($P<0.001$). Rats fed KO had a higher ($P<0.001$) final P urine excretion than

rats fed CO, FO, MO, SO, or TO (Table 10). Rats fed FO at baseline had a higher P absorption rate compared to KO (P=0.049), SO (P=0.03), or TO (P=0.002). Also at baseline, rats fed MO had a higher (P=0.005) P absorption rat compared to TO. Rat fed FO at final did not have a significantly different P absorption rate. At baseline, rats fed KO had a higher (P<0.001) P retention compared to CO, FO, MO, or TO. Also at baseline, rats fed SO had a higher P retention compared to CO (P=0.005), FO (P<0.001), MO (P=0.048), or TO (P=0.03) (Table 10). There was no significant difference in P retention at final.

5.7. Biochemical Measurements

There was no significant difference in serum Ca, P, alkaline phosphate, or PYD. Serum osteocalcin was higher in rats fed FO (P=0.03) compared to rats fed TO or SO. Rats fed SO or TO had lower serum TBARS than rats fed MO (P=0.004), KO (P=0.005), or CO (P=0.003). There were no significant differences in total antioxidants (Table 13). Serum TNF-α was below detectable levels (Table 13). Detection limit of TNF-α was 31.25 pg TNF-α /mL serum. Relative urinary PGE_2 resulted in no difference in FO compared to CO, but was lower in KO, MO, SO, or TO compared to CO and FO (Figure 5).

Table 3: Food Intake, Weight Gain, and Final Body Weight of Growing Female Rats Fed Different Sources of Omega-3 Polyunsaturated Fatty Acids[3]

Treatments	Total Food Intake (g)	Weight gain (g)	Final Body Weight (g)
CO	750.1 ± 12.5	75.99 ± 7.42	214.92 ± 6.36
FO	762.9 ± 14.3	103.75 ± 8.11	239.58 ± 8.24
KO	767.5 ± 10.9	98.39 ± 6.12	231.70 ± 6.58
MO	761.2 ± 17.6	107.60 ± 12.53	241.46 ± 14.39
SO	706.8 ± 17.4	85.97 ± 8.41	215.49 ± 10.27
TO	738.3 ± 16.4	106.02 ± 6.66	234.95 ± 8.19
***P* value**	0.06	0.06	0.19

[3] Values are expressed as the mean ± SEM of n=10 rats/group. Abbreviations are CO=corn oil, FO=flaxseed oil, KO=krill oil, MO=menhaden oil, SO=salmon oil, TO=tuna oil.

Table 4: Bone Morphometry of Growing Female Rats Fed Different Sources of Omega-3 Polyunsaturated Fatty Acids[4]

Treatments	Length (cm)	Depth (mm)	Width (mm)	Dry Weight (g)
Femur				
CO	3.16 ± 0.01	4.60 ± 0.14 ab	3.08 ± 0.17	0.47 ± 0.01
FO	3.21 ± 0.03	4.84 ± 0.12 b	3.08 ± 0.10	0.50 ± 0.01
KO	3.13 ± 0.01	4.36 ± 0.17 a	3.24 ± 0.16	0.47 ± 0.01
MO	3.14 ± 0.01	4.73 ± 0.06 ab	2.99 ± 0.04	0.50 ± 0.01
SO	3.13 ± 0.02	4.81 ± 0.08 ab	3.01 ± 0.03	0.48 ± 0.01
TO	3.18 ± 0.03	4.79 ± 0.07 ab	2.98 ± 0.03	0.48 ± 0.01
***P* value**	0.05	0.026	0.53	0.09
Tibia				
CO	3.55 ± 0.03 a	3.65 ± 0.06	2.62 ± 0.06	0.30 ± 0.01 a
FO	3.60 ± 0.03 a	3.83 ± 0.12	2.81 ± 0.09	0.34 ± 0.01 bc
KO	3.51 ± 0.04 a	3.67 ± 0.08	2.83 ± 0.07	0.32 ± 0.01 ab
MO	3.59 ± 0.03 a	3.80 ± 0.06	2.79 ± 0.08	0.35 ± 0.01 bc
SO	3.63 ± 0.03 ab	3.92 ± 0.06	2.88 ± 0.07	0.37 ± 0.01 c
TO	3.72 ± 0.02 b	3.87 ± 0.05	2.79 ± 0.07	0.38 ± 0.01 c
***P* value**	<0.001	0.07	0.27	<0.001

[4] Values are expressed as the mean ± SEM of n=10 bone pairs/group. Different letters a, b, c, within the same column indicate significant differences at $P<0.05$ by one-way ANOVA followed by Tukey's test. Abbreviations are CO=corn oil, FO=flaxseed oil, KO=krill oil, MO=menhaden oil, SO=salmon oil, TO=tuna oil.

Table 5: Bone Mineralization in Growing Female Rats Fed Different Sources of Omega-3 Polyunsaturated Fatty Acids[5]

Treatment	BMA (cm^2)	BMC (g)	BMD (mg/cm^2)	Ash Weight (g)	Ca (mg/g bone)	P (mg/g bone)
Femur						
CO	1.56 ± 0.02	0.33 ± 0.01	0.211 ± 0.003	0.294 ± 0.004	399.2 ± 8.2	187.0 ± 3.5
FO	1.55 ± 0.05	0.35 ± 0.02	0.225 ± 0.003	0.319 ± 0.007	413.9 ± 8.0	193.7 ± 3.0
KO	1.54 ± 0.03	0.34 ± 0.01	0.220 ± 0.003	0.303 ± 0.006	392.7 ± 11.8	183.9 ± 5.2
MO	1.56 ± 0.03	0.34 ± 0.01	0.220 ± 0.003	0.314 ± 0.005	401.7 ± 5.3	188.0 ± 1.8
SO	1.55 ± 0.04	0.33 ± 0.01	0.212 ± 0.004	0.298 ± 0.009	395.6 ± 7.6	185.9 ± 3.3
TO	1.58 ± 0.04	0.35 ± 0.01	0.219 ± 0.003	0.312 ± 0.007	392.6 ± 5.3	183.9 ± 2.1
***P* value**	0.98	0.79	0.08	0.06	0.42	0.31
Tibia						
CO	1.12 ± 0.01 a	0.19 ± 0.01 a	0.165 ± 0.002 a	0.189 ± 0.004 a	381.6 ± 5.2	181.7 ± 2.3
FO	1.21 ± 0.03 ab	0.21 ± 0.01 ab	0.177 ± 0.002 ab	0.216 ± 0.005 b	386.6 ± 7.0	183.3 ± 3.1
KO	1.17 ± 0.04 a	0.20 ± 0.01 ab	0.171 ± 0.003 ab	0.202 ± 0.005 a	391.3 ± 9.8	186.0 ± 4.4
MO	1.18 ± 0.02 a	0.21 ± 0.01 ab	0.176 ± 0.001 ab	0.216 ± 0.005 b	366.0 ± 16.2	178.6 ± 5.6
SO	1.28 ± 0.04 b	0.23 ± 0.01 b	0.176 ± 0.004 ab	0.217 ± 0.007 b	406.1 ± 6.8	190.0 ± 2.9
TO	1.32 ± 0.03 b	0.24 ± 0.01 b	0.181 ± 0.003 b	0.231 ± 0.004 b	396.6 ± 4.8	187.5 ± 1.9
***P* value**	<0.001	<0.001	0.009	<0.001	0.05	0.26

[5] Values are expressed as the mean ± SEM of n=7-10 bones/group. Different letters a, b, within the same column indicate significant differences at $P<0.05$ by one-way ANOVA followed by Tukey's test. Abbreviations are BMA=bone mineral area, BMC= bone mineral content, BMD= bone mineral density, Ca=calcium, P=phosphorus, CO=corn oil, FO=flaxseed oil, KO=krill oil, MO=menhaden oil, SO=salmon oil, TO=tuna oil.

Table 6: Trabecular Bone Microarchitecture of Growing Female Rats Fed Different Sources of Omega-3 Polyunsaturated Fatty Acids[6]

Treatment	BV/TV (%)	TbN (per mm)	TbTh (mm)	TbSp (mm)	CONN (1/mm^3)	SMI
Femur						
CO	8.0 ± 0.7 a	2.31 ± 0.15 a	0.059 ± 0.001	0.46 ± 0.03 b	34.30 ± 4.20 a	2.39 ± 0.06
FO	14.0 ± 1.0 b	3.28 ± 0.22 b	0.063 ± 0.001	0.32 ± 0.02 a	64.03 ± 5.07 b	2.16 ± 0.08
KO	11.0 ± 0.9 ab	2.65 ± 0.23 ab	0.065 ± 0.003	0.41 ± 0.03 ab	44.30 ± 5.51 ab	2.45 ± 0.07
MO	14.0 ± 0.7 b	3.18 ± 0.19 b	0.066 ± 0.002	0.33 ± 0.02 a	60.88 ± 5.63 b	2.19 ± 0.07
SO	11.0 ± 1.5 ab	2.57 ± 0.13 ab	0.063 ± 0.003	0.40 ± 0.02 ab	43.98 ± 7.09 ab	2.38 ± 0.12
TO	11.0 ± 0.8 ab	2.83 ± 0.17 ab	0.061 ± 0.001	0.38 ± 0.03 ab	46.49 ± 4.27 ab	2.43 ± 0.07
***P* value**	0.002	0.003	0.17	0.003	0.002	0.05
Tibia						
CO	13.06 ± 1.78 a	2.13 ± 0.29 a	0.076 ± 0.001 ab	0.52 ± 0.07 b	35.42 ± 7.88 a	2.32 ± 0.08 b
FO	23.23 ± 1.93 b	3.41 ± 0.27 b	0.082 ± 0.002 b	0.31 ± 0.03 a	73.50 ± 6.94 b	1.77 ± 0.12 a
KO	18.68 ± 1.57 ab	3.09 ± 0.25 ab	0.080 ± 0.002 ab	0.33 ± 0.29 a	59.04 ± 5.83 ab	2.17 ± 0.08 ab
MO	21.09 ± 2.29 ab	3.53 ± 0.27 b	0.081 ± 0.002 ab	0.28 ± 0.02 a	68.45 ± 9.85 b	2.03 ± 0.15 ab
SO	15.95 ± 1.59 a	2.70 ± 0.18 ab	0.075 ± 0.001 a	0.39 ± 0.03 ab	48.92 ± 6.95 ab	2.31 ± 0.12 b
TO	18.93 ± 1.42 ab	3.12 ± 0.15 ab	0.078 ± 0.001 ab	0.32 ± 0.02 a	58.91 ± 4.94 ab	2.09 ± 0.11 ab
***P* value**	0.007	0.003	0.020	<0.001	0.013	0.021

[6] Values are expressed as the mean ± SEM of n=9-10 bones/group. Different letters a, b, within the same column indicate significant differences at $P<0.05$ by one-way ANOVA followed by Tukey's test. Abbreviations are BV/TV= trabecular bone volume per unit of total volume, TbN=Trabecular number, TbTh=Trabecular thickness, TbSp=Trabecular space, CONN=Connectivity, SMI=Structure model index, , CO=corn oil, FO=flaxseed oil, KO=krill oil, MO=menhaden oil, SO=salmon oil, TO=tuna oil.

Table 7: Cortical Bone Microarchitecture of Growing Female Rats Fed Different Sources of Omega-3 Polyunsaturated Fatty Acids[7]

Treatment	BV/TV (%)	CtTh (mm)	Cortical Area (mm^2)	Porosity (%)
Femur				
CO	96.0 ± 0.1 b	0.548 ± 0.007	4.95 ± 0.07	4.02 ± 0.13
FO	95.1 ± 0.2 ab	0.548 ± 0.009	5.18 ± 0.11	4.86 ± 0.22
KO	95.4 ± 0.1 ab	0.542 ± 0.006	5.10 ± 0.13	4.64 ± 0.13
MO	95.0 ± 0.4 ab	0.538 ± 0.006	5.18 ± 0.12	5.04 ± 0.36
SO	95.0 ± 0.2 a	0.527 ± 0.009	5.24 ± 0.25	4.98 ± 0.22
TO	95.1 ± 0.4 ab	0.546 ± 0.008	5.15 ± 0.15	4.86 ± 0.39
***P* value**	0.024	0.33	0.77	0.07
Tibia				
CO	97.8 ± 0.1	0.538 ± 0.001	3.25 ± 0.04	2.18 ± 0.11
FO	97.9 ± 0.1	0.569 ± 0.001	3.58 ± 0.09	2.08 ± 0.13
KO	97.6 ± 0.1	0.553 ± 0.005	3.64 ± 0.07	2.32 ± 0.18
MO	97.6 ± 0.1	0.560 ± 0.007	3.58 ± 0.05	2.36 ± 0.11
SO	97.5 ± 0.2	0.556 ± 0.009	3.58 ± 0.11	2.46 ± 0.26
TO	97.8 ± 0.1	0.571 ± 0.008	3.58 ± 0.08	2.14 ± 0.09
***P* value**	0.57	0.09	0.07	0.51

[7] Values are expressed as the mean ± SEM of n=7-10 bones/group. Different letters a, b, within the same column indicate significant differences at $P<0.05$ by one-way ANOVA followed by Tukey's test. Abbreviations are BV/TV= trabecular bone volume per unit of total volume, CtTh=Cortical thickness, CO=corn oil, FO=flaxseed oil, KO=krill oil, MO=menhaden oil, SO=salmon oil, TO=tuna oil.

Table 8: Bone Strength in Growing Female Rats Fed Different Sources of Omega-3 Polyunsaturated Fatty Acids[8]

Treatment	Peak Force (N)	Ultimate Stiffness (N/S)	Ultimate Bending Stress (N/mm^2)	Young's Modulus (N/mm^2)	Bending Failure Energy (N.S)
Femur					
CO	119.4 ± 4.5	352.6 ± 34.1	125.3 ± 6.5	3535.0 ± 438.8	21.3 ± 1.5
FO	129.7 ± 5.1	391.9 ± 27.2	126.0 ± 5.6	3587.5 ± 312.9	42.5 ± 17.3
KO	123.4 ± 5.1	395.3 ± 26.3	124.0 ± 4.4	3548.1 ± 242.4	20.5 ± 1.5
MO	130.5 ± 4.3	378.0 ± 31.1	133.6 ± 4.5	3722.3 ± 333.0	26.4 ± 1.4
SO	128.3 ± 6.0	435.9 ± 29.6	127.1 ± 5.0	4054.3 ± 265.0	22.0 ± 1.9
TO	129.3 ± 6.0	424.4 ± 28.2	131.7 ± 5.7	4128.8 ± 293.4	22.2 ± 1.9
***P* Value**	0.61	0.39	0.76	0.64	0.24
Tibia					
CO	81.1 ± 5.6	264.5 ± 27.8	131.0 ± 8.8	4558.5 ± 412.9	16.7 ± 1.5
FO	89.8 ± 6.0	312.1 ± 36.7	128.1 ± 10.6	4756.9 ± 781.0	16.9 ± 1.1
KO	92.2 ± 5.8	263.8 ± 29.3	130.9 ± 6.5	3760.0 ± 382.5	21.7 ± 2.0
MO	93.5 ± 5.0	260.6 ± 32.1	136.2 ± 11.7	3902.2 ± 600.8	23.1 ± 2.6
SO	98.4 ± 4.9	351.6 ± 26.8	129.3 ± 7.6	4762.0 ± 549.8	18.4 ± 2.0
TO	105.7 ± 6.1	323.5 ± 19.7	148.0 ± 9.3	4720.4 ± 396.0	20.8 ± 1.8
***P* Value**	0.07	0.13	0.66	0.62	0.09

[8] Values are expressed as the mean ± SEM of n=9-10 bone pairs/group. Abbreviations are CO=corn oil, FO=flaxseed oil, KO=krill oil, MO=menhaden oil, SO=salmon oil, TO=tuna oil.

Table 9: Ca Intake, Excretion, Absorption, and Retention of Growing Female Rats Fed Different Sources of Omega-3 Polyunsaturated Fatty Acids[9]

Treatment	Ca Intake (mg/d)	Fecal Ca Excretion (mg/d)	Urinary Ca Excretion (mg/d)	Ca Absorption* Rate (%)	Ca Retention† (mg/d)
Baseline					
CO	78.479 ± 3.407	20.683 ± 1.969 bc	0.269 ± 0.070	72.596 ± 3.323 a	69.056 ± 7.221
FO	80.006 ± 3.171	12.996 ± 0.945 a	0.339 ± 0.079	83.620 ± 1.181 b	71.283 ± 3.354
KO	80.000 ± 3.566	18.339 ± 1.874 ab	0.236 ± 0.074	76.715 ± 2.525 ab	69.515 ± 5.441
MO	78.876 ± 3.772	12.636 ± 0.766 a	0.179 ± 0.063	83.507 ± 1.436 b	73.966 ± 5.188
SO	82.300 ± 1.075	26.464 ± 2.049 c	0.330 ± 0.065	68.019 ± 2.159 a	59.112 ± 2.183
TO	83.786 ± 1.448	27.502 ± 2.102 c	0.327 ± 0.073	67.207 ± 2.382 a	57.125 ± 1.907
***P* Value**	0.78	<0.001	0.54	<0.001	0.06
Final Week					
CO	86.799 ± 4.403 b	45.373 ± 6.590	0.671 ± 0.298	48.589 ± 6.455	40.754 ± 5.459
FO	71.883 ± 1.899 ab	30.018 ± 1.765	0.575 ± 0.127	58.304 ± 2.206	41.291 ± 1.786
KO	82.090 ± 4.064 ab	47.251 ± 3.682	0.507 ± 0.212	40.616 ± 5.755	34.332 ± 6.023
MO	77.021 ± 4.102 ab	31.157 ± 3.775	0.668 ± 0.209	60.208 ± 3.715	45.195 ± 2.952
SO	77.855 ± 4.291 ab	37.903 ± 4.522	0.216 ± 0.041	50.716 ± 5.774	39.735 ± 5.279
TO	70.909 ± 2.917 a	35.624 ± 2.947	0.458 ± 0.335	49.204 ± 4.282	34.828 ± 3.805
***P* Value**	0.034	0.019	0.73	0.08	0.52

[9] Values are expressed as the mean ± SEM of n=10 rats/group

* Ca absorption rate (%) = (Ca intake – Fecal Ca excretion) / Ca intake x 100; Δ Ca absorption = Final week – Baseline week

† Ca retention = Total Ca Intake – (Fecal Ca excretion + Urinary Ca excretion); Δ Ca retention = Final week – Baseline week

Abbreviations are Ca=calcium, CO=corn oil, FO=flaxseed oil, KO=krill oil, MO=menhaden oil, SO=salmon oil, TO=tuna oil.

Table 10: P Intake, Excretion, Absorption, and Retention of Growing Female Rats Fed Different Sources of Omega-3 Polyunsaturated Fatty Acids[10]

Treatment	P Intake (mg/d)	Fecal P Excretion (mg/d)	Urinary P Excretion (mg/d)	P Absorption* Rate (%)	P Retention† (mg/d)
Baseline					
CO	47.354 ± 2.056 a	7.899 ± 0.485 ab	3.854 ± 0.934 ab	82.631 ± 1.680 abc	35.601 ± 2.724 a
FO	45.323 ± 1.796 a	5.315 ± 0.501 a	6.377 ± 1.278 b	88.686 ± 1.239 b	33.631 ± 2.950 a
KO	78.313 ± 3.491 c	8.302 ± 0.905 b	3.466 ± 0.474 ab	82.224 ± 2.253 ac	66.545 ± 3.683 b
MO	47.137 ± 2.254 a	5.494 ± 0.246 a	3.128 ± 0.509 a	88.104 ± 0.814 bc	38.515 ± 2.142 a
SO	59.249 ± 0.774 b	8.968 ± 0.670 b	1.500 ± 0.205 a	81.889 ± 1.258 ac	48.781 ± 0.790 b
TO	49.482 ± 0.855 a	10.198 ± 0.892 b	1.550 ± 0.186 a	79.764 ± 1.617 a	37.734 ± 0.997 a
***P* Value**	<0.001	<0.001	<0.001	<0.001	<0.001
Final Week					
CO	36.506 ± 1.852 a	17.738 ± 2.144 ab	4.330 ± 1.864 a	51.616 ± 5.175	14.438 ± 3.402
FO	34.955 ± 0.923 a	12.589 ± 0.723 a	7.533 ± 1.735 a	63.987 ± 1.964	14.833 ± 1.919
KO	62.923 ± 3.115 c	24.218 ± 1.789 b	21.316 ± 2.065 b	60.421 ± 3.609	17.389 ± 5.049
MO	36.272 ± 1.932 a	13.331 ± 1.492 a	4.176 ± 1.311 a	63.482 ± 3.320	18.765 ± 2.128
SO	47.756 ± 2.632 b	18.992 ± 2.448 ab	2.647 ± 0.323 a	59.634 ± 5.007	26.117 ± 3.247
TO	39.219 ± 1.613 ab	16.363 ± 1.293 a	3.197 ± 0.642 a	57.810 ± 3.391	19.659 ± 1.736
***P* Value**	<0.001	<0.001	<0.001	0.26	0.12

[10] Values are expressed as the mean ± SEM of n=10 rats/group

* P absorption rate (%) = (P intake – Fecal P excretion) / P intake x 100; Δ P absorption = Final week – Baseline week

† P retention = Total P Intake – (Fecal P excretion + Urinary P excretion); Δ P retention = Final week – Baseline week

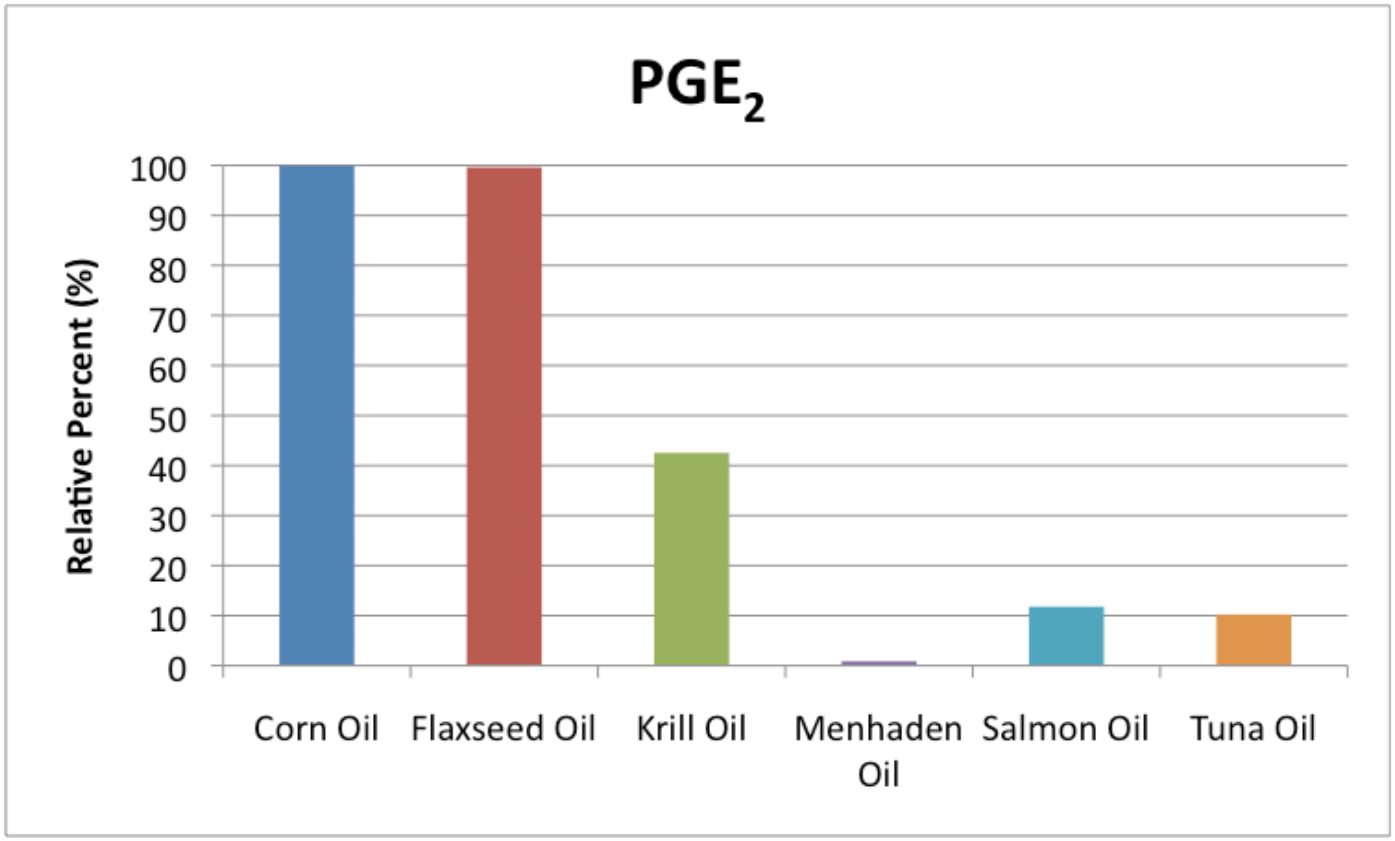
Values are expressed as the mean ± SEM of n=10 rats/group. Different letters a, b, within the same column indicate significant differences at $P<0.05$ by one-way ANOVA followed by Tukey's test. Abbreviations are P=phosphorous, CO=corn oil, FO=flaxseed oil, KO=krill oil, MO=menhaden oil, SO=salmon oil, TO=tuna oil.

Table 11: Serum Measurements of Bone Turnover and Oxidative Stress of Growing Female Rats Fed Different Sources of Omega-3 Polyunsaturated Fatty Acids[11]

Treatment	Ca (mg/dL)	P (mg/dL)	ALP (U/L)	PYD (nmol/L)	Osteocalcin (ng/µL)	TBARS (µM MDA)	Total Antioxidants (mM Trolox)
CO	15.2 ± 1.1	10.7 ± 0.7	261.8 ± 39.5	6.735 ± 0.904	176.0 ± 27.0 ab	6.9 ± 0.4 b	1.5 ± 0.2
FO	14.0 ± 1.0	10.5 ± 0.4	223.5 ± 50.0	6.597 ± 0.497	164.5 ± 23.4 b	5.9 ± 0.2 ab	1.1 ± 0.1
KO	13.0 ± 1.1	10.5 ± 0.7	195.4 ± 24.9	7.146 ± 0.531	132.1 ± 11.0 ab	6.5 ± 0.3 b	1.3 ± 0.1
MO	13.7 ± 0.5	10.6 ± 0.6	163.1 ± 17.0	5.985 ± 0.295	131.2 ± 16.1 ab	6.8 ± 0.3 b	1.5 ± 0.2
SO	13.0 ± 0.5	9.8 ± 0.5	170.3 ± 18.2	7.398 ± 0.454	136.5 ± 8.0 a	5.1 ± 0.3 a	1.1 ± 0.2
TO	12.6 ± 0.3	10.1 ± 0.2	167.0 ± 17.1	7.103 ± 0.514	107.6 ± 15.3 a	5.2 ±0.2 a	1.2 ± 0.2
***P* Value**	0.24	0.83	0.19	0.55	0.015	0.005	0.28

[11] Values are expressed as the mean ± SEM of n=8-10 rats/group. Different letters a, b, within the same column indicate significant differences at $P<0.05$ by one-way ANOVA followed by Tukey's test. Abbreviations are Ca=calcium, P=Phosphorus, ALP=Alkaline phosphatase, TBARS= Thiobarbituric acid reactive substances, PYD=Pyridinoline, CO=corn oil, FO=flaxseed oil, KO=krill oil, MO=menhaden oil, SO=salmon oil, TO=tuna oil.

Figure 5: Relative Percent of Urine Prostaglandin E_2 [12]



[12] Abbreviations are: PGE_2=prostaglandin E_2, CO=corn oil, KO=krill oil, MO=menhaden oil, FO=flaxseed oil, TO=tuna oil, SO=salmon oil

6.0. Discussion

This study demonstrated that certain sources of ω-3 PUFAs have a positive effect on the long bone of growing female rats fed a high (12%) fat diet. The results showed that the different sources of ω-3 PUFAs had different effects on bone morphometry. TO-fed rats had a greater tibia longitudinal growth compared to all groups except SO fed rats (P=0.03). In our study, TO followed by SO were the richest sources of DHA among the different sources of ω-3 PUFA. Reinwald et al. (2004) reported increased (P=0.03) tibiae length in weanling female and male Long-Evan rats fed an essential fatty acid deficient diet supplemented with DHA for 8 weeks compared to those fed an ω-3 PUFA adequate diet. Li et al. (2010) fed Long-Evans pregnant rats a preformed docosapentaenoic acid (DPA, 22:5 ω-6) to yield ω-3 deficient pups. The pups were then fed a diet of DHA, DPA, or DHA plus DPA. The authors found that DHA accumulated in the osteoblast-rich periosteum of the bone. Therefore, in our study, the mechanism for greater tibia length in TO-fed rats may be due to DHA activating osteoblasts to have been shown to promote greater bone growth. Rats fed KO had reduced (P=0.03) femoral depth than rats fed FO. Additionally, tibia weight was lighter in rats fed KO compared to rats fed SO (P=0.007) and TO (P=0.002). KO is high in phospholipids, and oxidized phospholipids promoted osteoclast activity (Tseng et al., 2010), which may lead to the lighter and thinner bones observed in this study.

In addition to changes in bone morphometry, feeding different sources of ω-3 PUFAs to female growing rats affected bone mineralization. Tibial BMD was higher (P=0.009) in TO-fed rats compared to rats fed CO. Tibial BMA (P<0.001) and BMC (P<0.001) were also higher in rats fed TO as well as SO compared to rats fed CO.

There was no effect of ω-3 PUFA supplementation on femur mineralization. Sirois et al. (2003) reported female Sprague-Dawley rats fed either a diet consisting of soybean oil, rich in ALA, or menhaden oil, rich in DHA, for 5 weeks also had no effect on femoral BMC and BMD. In a restricted-feeding (50% calorie reduction in food) rat study (Sun et al., 2004), female Wistar rats that consumed DHA showed no change in femoral BMD. In our study, changes in bone mineralization occurred in the tibiae, but not in the femur may be due to the tibiae having a higher turnover rate than femurs (Wallace, 2007). The tibiae have a higher turnover rate because the tibiae are subjected to more complex loading state than femurs (Wallace, 2007). In a human study of adolescent white males (average age 16.7 years), a higher ratio ω-6:ω-3 PUFA intake showed a negative correlation to spine BMD (r= -0.26, P=0.02); whereas, serum DHA and ω-3 PUFA concentrations were positively correlated to spine (r=0.30, P=0.008) and total body BMD (r=0.32, P=0.004) (Hogstrom, 2007). The results suggested consumption of ω-3 PUFA, especially DHA, increased bone mineralization during growth. In our study, TO, the richest source of DHA, promoted bone mineralization to a greater extent than the other sources of ω-3 PUFA.

The current study also included measurement of cortical and trabecular bone. Cortical microarchitecture was not affected by feeding different sources of ω-3 PUFAs with the exception of lower (P=0.06) femur cortical BV/TV in rats fed SO compared to rats fed CO. Reinwald et al. (2004), reported that femoral cortical bone was not as responsive to ω-3 PUFA repletion in rats fed an essential fatty acid deficient diet. In contrast, trabecular bone is more metabolically active and undergoes more rapid remodeling than cortical bone (Watts, 1999). In the current study, MO and particularly FO appeared to be the sources of ω-3 PUFA that improved bone microarchitecture quality. Rats fed FO or MO had increased tibia trabecular bone indicated by

higher BV/TV (P=0.007) and trabecular thickness (P=0.02) than rats fed CO and SO. Rats fed FO or MO had increased tibia TbN (P=0.003) and CONN (P=0.01) compared to rats fed CO. FO followed by MO had the highest amount of ALA compared to the other oils. Therefore improved microarchitecture in the bones of growing female rats may be due to the ALA.

Maximizing bone mineralization and bone matrix should result in stronger bones. Despite microarchitecture and mineralization differences in rats fed different sources of ω-3 PUFAs in our study, there were no significant differences in biomechanical strength. Similarly, Kruger et al. (2005) reported that rats fed either corn, evening primrose, or tuna oil for 6 weeks had no significant differences in bone biomechanical strength. Absence of strength differences in our study may be due to different effects of different sources of ω-3 PUFAs on bone. TO and SO increased bone mineralization and FO and MO increased trabecular microarchietecture quality, yet no group did both. Since both mineralization and microarchitecture is important for bone strength, oil that only improves one aspect will not improve bone strength. However maximizing bone mineralization and bone matrix during growth may preserve against future age-related bone loss leading to risk of osteoporosis (Cashman, 2007).

The study results suggested that different sources of ω-3 PUFAs may act differently on bone. Of the different sources of ω-3 PUFAs, rats fed TO and SO increased bone mineralization and rats fed MO and FO increased trabecular microarchitecture quality. Potential mechanisms of actions of the different sources of ω-3 PUFAs on bone health were investigated.

Indirectly, dietary fats may affect bone by altering Ca balance. Kruger et al. (2005) reported that young (age 5 weeks) male Sprague-Dawley rats fed 5% tuna oil g/kg diet had significantly higher Ca absorption and decreased urinary Ca compared to the rats fed evening primrose oil. The authors suggested that cell membrane saturation reduced Ca absorption. The

then decreased cell membrane saturation due to ω-3 PUFA consumption resulted in increased Ca absorption and decreased urinary Ca loss. Our study showed feeding TO had no significant effect on final Ca absorption or urinary excretion. Compared to the other sources of ω-3 PUFA Kruger et al. (2005) also suggested that essential fatty acids LA or ALA might affect Ca retention. Claassen et al., (1995a) fed growing (age 21 days) male Sprague-Dawley rats 3:1 gamma-LA:EPA decreased Ca excretion and had higher (P<0.05) bone Ca content compared to rats fed 1:3 gamma-LA:EPA. In our study, there was no difference in bone Ca content among any of the different sources of ω-3 PUFA. A mineral balance change observed was KO-fed rats had a greater final P urine excretion (P<0.001) compared to the other groups and a greater final P fecal excretion (P<0.001) compared to rats fed FO, MO, or TO. This may be due to the high P content associated with the high phospholipid content in KO (Gigliotti et al. 2010). However, these changes in P balance did not result in higher bone P in rats fed KO.

Another mechanism whereby ω-3 PUFAs may affect bone is by influencing oxidative stress. Oxidative stress may increase pro-inflammatory cytokines, which lead to an uncoupling of bone remodeling in favor of bone resorption (McLean et al., 2009). In the current study, rats fed TO or SO have reduced lipid oxidation indicated by lower (P<0.005) serum TBARS compared to rats fed CO, KO, or MO. PGE_2, a pro-inflammatory eicosanoid, showed lower relative urinary PGE_2 in rats fed SO or TO compared to CO. TNF-α, a pro-inflammatory cytokine, was below detectable levels in this study. The combination of reduced lipid peroxidation and pro-inflammation may have contributed to higher bone mineralization in rats fed TO or SO compared to CO. Rats fed FO showed no differences in serum TBARS compared to CO, but showed improved tibia trabecular microarchitecture. Bones weaken when osteoclast remove bone faster than osteoblasts deposit bone (Martini 2006). Claassen (1995b) reported

lower PYD levels in groups with higher levels of EPA and DHA compared to the control of a LA:ALA ratio of 3:1. In our study, there was no significant difference in PYD, an indicator of osteoclast activity, between the groups fed different sources of ω-3 PUFAs. However rats fed FO showed bone formation indicated by no differences in serum PYD and higher serum osteocalcin, an indicator of bone formation, compared to rats fed SO and TO. It is important to note that the bone turnover markers were taken at a single time point; therefore it is more difficult to make generalizations based on this data.

A potential mechanism whereby ω-3 PUFAs may influence bone formation is by altering the synthesis of PG. The essential fatty acid linoleic acid (LA, 18:2 ω-6) can be metabolized to AA and then PGE_2. A high concentration of PGE_2 inhibits bone formation (Mollard et al., 2005). FO is high in ALA, an ω-3 PUFA that competes with LA for the delta-6 desaturase. This makes ALA a competitor to the production of PGE_2. Therefore, consumption of FO, ALA competes with LA for delta-6 desaturase, potentially resulting in decreased enzymes for conversion of LA to AA. AA is a substrate for the synthesis of pro-inflammatory eicosanoids. Therefore, competitive inhibition of ALA with LA may result in reduced inflammation. In the current study, relative urinary PGE_2 was not significantly different between FO and CO. However, there are various other eicosanoids as well as cytokines involved in the inflammatory response.

7.0. Summary and Conclusion

The current study found that the source of ω-3 PUFAs is important to bone metabolism. Rats fed FO or MO, with the highest ALA content of the sources, ω-3 PUFAs showed improved bone microarchitecture and increased bone formation; whereas rats fed SO or TO, with the highest DHA of the sources, ω-3 PUFAs improved bone mineralization associated with a reduction in lipid oxidation. There was no difference in biomechanical strength in any of the groups. KO-fed rats did not appear to improve bone health compared to CO control indicated by no change in P or Ca content in bones and microarchitecture. This may be due to the PUFA in KO being in a phospholipid rather than TAG form.

The study results suggest that rather than focusing on one source of ω-3 PUFAs, perhaps a variety of sources of ω-3 PUFAs should be consumed in order to improve bone health during the bone growth stage.

8.0. References

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